

Preferential Expulsion of Dividing Algal Cells as a Mechanism for Regulating Algal-Cnidarian Symbiosis

GAREN BAGHDASARIAN* AND LEONARD MUSCATINE

*Department of Organismic Biology, Ecology, and Evolution, University of California, Los Angeles,
Los Angeles, California 90095-1606*

Abstract. A wide range of both intrinsic and environmental factors can influence the population dynamics of algae in symbiosis with marine cnidarians. The present study shows that loss of algae by expulsion from cnidarian hosts is one of the primary regulators of symbiont population density. Because there is a significant linear correlation between the rate of algal expulsion and the rate of algal division, factors that increase division rates (*e.g.*, elevated temperature) also increase expulsion rates. Additionally, ^3H -thymidine is taken up to a greater extent by algae destined to be expelled than by algae retained in the host cnidarians. Taken together, data for rates of expulsion, rates of division at different temperatures, and uptake of ^3H -thymidine suggest that dividing algal cells are preferentially expelled from their hosts. The preferential expulsion of dividing cells may be a mechanism for regulation of algal population density, where the rate of expulsion of algae may be an inverse function of the ability of host cells to accommodate new algal daughter cells. This kind of regulation is present in some cnidarian species (*e.g.*, *Aiptasia pulchella*, *Pocillopora damicornis*), but not in all (*e.g.*, *Montipora verrucosa*, *Porites compressa*, and *Fungia scutaria*).

Introduction

Algal-cnidarian symbioses are characterized by long-term stability wherein neither partner outgrows the other, and where algal population densities remain relatively constant (Drew, 1972; Pardy, 1974; Davies, 1984). During repopulation of aposymbiotic sea anemones (recovery from bleaching), symbiotic dinoflagellates grow at relatively high rates normally associated with log phase growth in culture (Berner *et al.*, 1993). If these high growth rates are sus-

tained, host fitness is reduced (Smith, 1992), leading to the eventual breakdown of the symbiosis (Neckelmann and Muscatine, 1983; Taylor *et al.*, 1989). Instead, as the size of the algal population reaches some optimum level, its growth rate decreases by a factor of 20 (Kinzie, 1974; Kinzie and Chee, 1979; Berner *et al.*, 1993), and a "steady state" is achieved, in which the growth rates of the algae and the host cells are in dynamic equilibrium. Clearly, regulation of symbiont population density is essential in maintaining a stable symbiosis, yet little is known of the cellular mechanisms involved.

A number of intrinsic and environmental factors can potentially regulate algal cell division and population growth. These factors could act pre- or post-mitotically (Hoegh-Guldberg and Smith, 1989); that is, population growth rate could be regulated either before or after algal cell division. Algal numbers could be regulated pre-mitotically by limited nutrient availability (see, for example, Blank and Muscatine, 1987; Kolber *et al.*, 1988; Falkowski *et al.*, 1993; Hoegh-Guldberg, 1994; Muller-Parker *et al.*, 1994; Snidvongs and Kinzie, 1994); by density-dependent negative feedback by the algae themselves (Muscatine and Pool, 1979; McAuley and Darrah, 1990); by host-induced release of photosynthate from the algae (Muscatine, 1967; Cook, 1983; Douglas and Smith, 1984; Sutton and Hoegh-Guldberg, 1990; McAuley, 1992; Gates *et al.*, 1995); or by factors manifested by the host cells that inhibit the algal cell cycle (Smith and Muscatine, 1999). Algal numbers could also be regulated postmitotically by degradation of algae *in situ* (Muscatine and Pool, 1979; Titlyanov *et al.*, 1996; Jones and Yellowlees, 1997), by direct expulsion of excess algae (Hoegh-Guldberg and Smith, 1989; Stimson and Kinzie, 1991; McCloskey *et al.*, 1996; Jones and Yellowlees, 1997), or by accommodation of excess algae by division of host cells (Muscatine and Pool, 1979; Neckelmann and

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* To whom correspondence should be addressed.

Muscatine, 1983; Smith and Muscatine, 1986; Titlyanov *et al.*, 1996).

Whereas previous studies have addressed pre-mitotic control of algae in symbiotic cnidarians, there is little information on mechanisms involved in post-mitotic regulation, especially relating to loss of algae by expulsion. Jones and Yellowlees (1997) showed that the combined effect of changes in rates of algal division and loss are involved in repopulation of bleached corals and in the eventual regulation of steady-state algal-cnidarian symbioses. In a previous study, we observed that the Hawaiian sea anemone *Aiptasia pulchella* maintained in the laboratory expels algae at a rate of about 0.046 d^{-1} (Baghdasarian and Muscatine, unpubl. data), a value high enough to be a major factor in regulating algal densities. Studies of other cnidarians report not only that algae are lost by expulsion, but also that the expelled algae have higher mitotic indices than algae retained by the host (Subarsono and Brown, 1992; McCloskey *et al.*, 1996). It has been hypothesized that the higher mitotic indices of expelled algae are due to release from some regulatory constraint by the hosts. In this study we test an alternative hypothesis, that higher mitotic indices of expelled algae are the result of preferential expulsion of dividing cells. It is important to note here that the release mechanism probably entails detachment of host cells (Gates *et al.*, 1992), their disintegration, and release of algae. Whereas the mechanism of preferential detachment of host cells remains to be addressed, here we focus on algal parameters.

Materials and Methods

Collection and maintenance of organisms

Specimens of the sea anemone *Aiptasia pulchella* Carlgren (1943) symbiotic with the dinoflagellate *Symbiodinium pulchrorum* were collected from Kaneohe Bay, Oahu, Hawaii (spring and fall 1995/1996). The animals were transferred by air to the University of California, Los Angeles, and maintained in natural seawater in 1.5-liter glass bowls in a Percival model I-35VL incubator at 25°C on a 12-h light/dark cycle at irradiance levels of $80\text{--}100 \mu\text{mole photons m}^{-2} \text{ s}^{-1}$, from two Rainbow Lifeguard 40-watt Primetinic and two General Electric 40-watt Cool White light sources. The animals were fed twice a week in the evenings on *Artemia* sp. The morning after every feeding, the bowls were cleaned using cotton swabs, and the seawater, collected from Redondo Beach, California, was replaced. Prior to the experiments, sea anemones were starved for 24 h under the light and temperature conditions described above.

The symbiotic corals *Porites compressa* Dana 1846, *Montipora verrucosa* Lamarek 1816, *Pocillopora damicornis* Linnaeus 1758, and *Fungia scutaria* Lamarek 1801 were collected from Kaneohe Bay, Oahu, Hawaii (October/November 1996). The corals were transferred to the laboratory

of the Hawaii Institute of Marine Biology, and were maintained in running seawater and exposed to natural light levels. All experiments were performed within 24 h of the collections.

Mitotic index of algae

Five *A. pulchella* were homogenized individually and their symbiotic algae isolated by repetitive centrifugation and resuspension (Steen, 1987). The technique was applied rigorously to eliminate the possibility of inclusion of algae still within host cells. The algal mitotic index (MI), defined as the percentage of cells with division plates (Wilkerson *et al.*, 1983) was determined by examination of at least 1000 algal cells under $400\times$ magnification using an Olympus BH-2 microscope. All additional microscopic observations were made under the same specifications.

Incorporation of ^3H -thymidine by algae in situ

Each of five *A. pulchella* specimens was incubated individually in 4 ml of a $2\text{-}\mu\text{Ci/ml}$ solution of ^3H -thymidine (Sigma Chemical Company; Sp. act. 50 Ci per mmol) in filtered seawater (FSW) for 24 h under the same maintenance conditions as the stock animals. The sea anemones were then washed serially 10 times in FSW to remove unincorporated ^3H -thymidine. Each wash consisted of adding fresh FSW, irrigating the coelenteron with a Pasteur pipette, and then waiting 3 min before changing the water again. Seven to nine washes were sufficient to remove unincorporated ^3H -thymidine from the sea anemones and the incubation medium. Algae expelled during the labeling period were discarded. ^3H -thymidine-labeled sea anemones were then incubated in FSW for an additional 15.5 h at 25°C in the light. At the end of the incubation period, both the algae released and those remaining in the hosts were isolated (using techniques described above on ice), adjusted to known volumes, and counted with a hemacytometer (Fisher Scientific). One-hundred-microliter samples of algae, along with 5 ml of Bio-Safe II biodegradable counting cocktail, were then added to plastic scintillation vials, and the incorporation of ^3H -thymidine by the algae was determined using an LKB Wallac 1214 Rackbeta liquid scintillation counter. Results were expressed as $\text{DPM} * (10^6 \text{ algae})^{-1}$. To determine whether the incorporation of ^3H -thymidine was by the algae or by host cells that contain the algae and may have been released along with them, techniques described in Gates and Muscatine (1992) were used to stain cells with Hoechst 33258 to check for the occurrence of host cell nuclei.

Rates of expulsion of algae

Fourteen sea anemones were allowed to settle in 4 ml of FSW in 15-ml test tubes. Six of the sea anemones were

incubated for 15.5 h at 25°C in the light, and the other eight were incubated for 15.5 h at 27.5°C in the light. After the incubation, algae in the incubation medium were recovered by centrifugation and set aside for analysis. Algae remaining in the animals were isolated by homogenization and centrifugation (Steen, 1987). Both the algae expelled into the medium and those retained by the hosts were counted, and the MI of the expelled algae was determined (Hoegh-Guldberg *et al.*, 1987).

Mitotic index and rate of expulsion of algae in corals

Mitotic indices and rates of algal expulsion were also established for four species of scleractinian corals commonly found in Kaneohe Bay, Oahu, Hawaii. Six pieces of each coral type from different colonies (*P. compressa*, *M. verrucosa*, *P. damicornis*, and in the case of *F. scutaria*, entire corals) were incubated in 30 ml of FSW at 27°C (seawater temperature in Kaneohe Bay) for 15.5 h. Next, to determine the effect of slight elevations of temperature on MI values and algal expulsion rates, six additional pieces from each coral type were incubated at 29.5°C for the same length of time. At the end of the incubation period, the corals were removed from the seawater. The released algae were collected by high-speed centrifugation of the incubation medium using a Damon IEC clinical centrifuge, followed by resuspension in 5 ml of fresh FSW. Algae remaining in the corals were removed with a toothbrush. The product was collected in FSW. The algae were then cleaned of mucus and animal tissue by centrifugation and resuspension in 20 ml of FSW, and MI values and rates of algal expulsion were determined using the techniques described above.

Results

Mitotic index of algae: natural expulsion vs. mechanical isolation

Algae naturally expelled from *A. pulchella* have a higher MI than the algae remaining in the hosts (Fig. 1; Wilcoxon signed rank nonparametric test: $P = 0.001$). To determine if the higher MI of the expelled algae is due to the absence of host-related regulation, algae were mechanically isolated from the sea anemones, and the MI was measured immediately and after a 15.5-h incubation at 25°C in the light. No significant changes in MI were observed following the 15.5-h incubation period (Wilcoxon signed rank nonparametric test: $P = 0.593$). These data suggest that the higher MI of algae expelled by *A. pulchella* is not necessarily due to release from putative host-related regulation.

Incorporation of ^3H -thymidine by algae in situ

To determine if the higher MI of expelled algal cells is due to preferential expulsion of dividing algae, sea anemones were incubated with ^3H -thymidine for 24 h, rinsed free of unincorporated ^3H -thymidine, and then incubated in

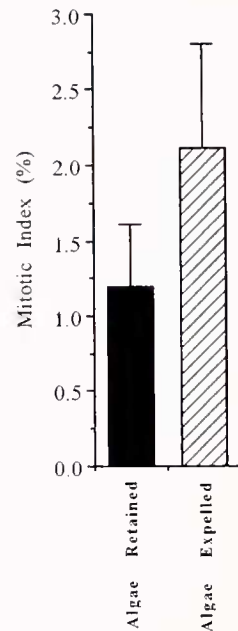


Figure 1. Mitotic index of the symbiotic alga *Symbiodinium pulchrum* after a 15.5-h incubation of the host sea anemone, *Aiptasia pulchella*, at 25°C. Comparison between expelled algae (▨) and algae retained by the host (■). Error bars represent standard deviations of the mean.

FSW for 15.5 h. Algae expelled and algae retained in the hosts were then assayed for incorporation of ^3H -thymidine. Expelled algae had incorporated significantly higher levels of ^3H -thymidine than cells remaining in the hosts (Fig. 2; Wilcoxon signed rank nonparametric test: $P = 0.043$). Because released algae are often contained within host cells (Gates *et al.*, 1992), it was important to determine whether the ^3H was associated with the algae or with the nuclei of host cells. This question was investigated by staining samples of retained and released algae with Hoechst 33582 to detect host cell nuclei that might be associated with the algae. Using epifluorescence microscopy to analyze the cells (Gates *et al.*, 1992), we found no evidence of host cell nuclei (*i.e.*, host nuclear DNA contamination). Taken together, these data suggest that the host preferentially expels algal cells that have entered S-phase of the cell cycle.

Mitotic index and rate of expulsion of algae

The correlation between the MI of the expelled algae and the rate of expulsion of these cells from the host could distinguish between expulsion of algae in random phases of the cell cycle *versus* expulsion of algae in a preferred phase of the cell cycle. If expulsion of algae from the host is random, then there should be no correlation between division rate of the expelled algae (*i.e.*, MI) and rate of expulsion. If, however, expulsion of algae is a function of cell cycle phase (more specifically, preferential release during late G_2 or M phases), then a positive correlation between the two parameters would be expected, as illustrated

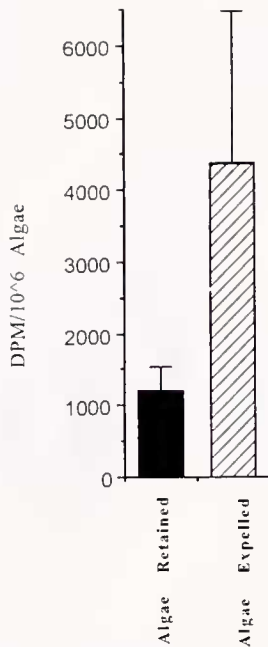


Figure 2. ³H-Thymidine incorporation by the symbiotic alga *Symbiodinium pulchrorum* after a 15.5-h incubation of the host sea anemone, *Aiptasia pulchella*, in filtered seawater. Comparison between expelled algae (▨) and algae retained by the host (■). Sea anemones were initially incubated in a 2- μ Ci/ml solution of ³H-thymidine for 24 h. Error bars represent standard deviations of the mean.

theoretically in Figure 3. Figure 4 shows that there is a positive linear correlation between expulsion rate of algae and their MI.

Effect of temperature on rate of algal expulsion

If expulsion of algae is affected by algal division rate, then environmental factors (such as slight increases in seawater temperature) that increase algal division rate (and hence MI) should also increase rate of expulsion of algae. Slightly elevated temperatures resulted in higher MI and expulsion rates (Fig. 5; Wilcoxon signed rank nonparametric test; Expulsion rate: $P = 0.034$, MI: $P = 0.050$). Further, the ratio of algal expulsion to MI at 25°C = 2.2, and at 27.5°C = 2.4. The similarity of these two ratios suggests that the higher MI values due to slight elevations in temperature are concomitant with higher algal expulsion rates. Finally, at 27.5°C, the observed changes in rates of algal division and expulsion follow the same positive linear correlation associated with preferential algal expulsion as a function of their MI (Fig. 6).

Rates of algal expulsion in corals

The relation between algal expulsion rate and division rate was investigated in four species of Hawaiian corals. In general, the released algae had a higher MI than did the algae remaining in the hosts (Fig. 7; Wilcoxon signed

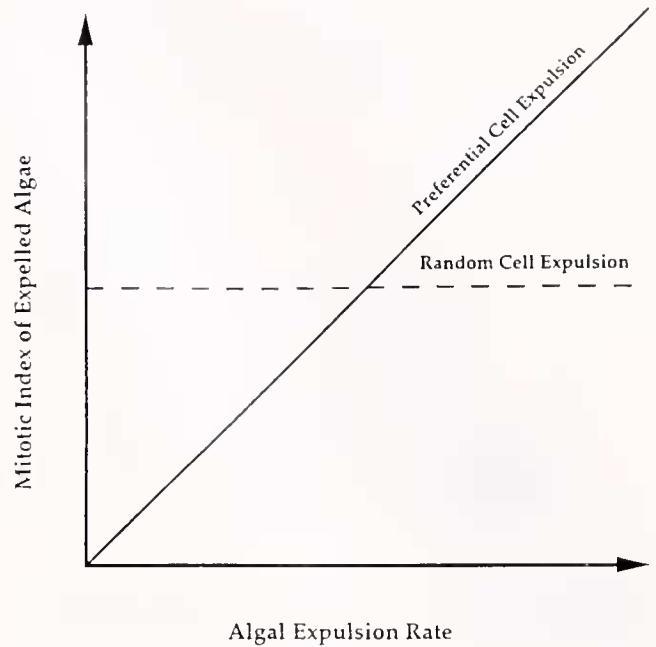


Figure 3. Hypothetical correlation between mitotic index of expelled symbiotic algae and their rate of expulsion from the hosts under conditions of preferential versus random cell expulsion.

rank nonparametric test; significant differences seen in *Pocillopora damicornis*, *Montipora verrucosa*, *Fungia scutaria*: $P = 0.028$, but not in *Porites compressa*: $P = 0.249$). However, the linear correlation between algal

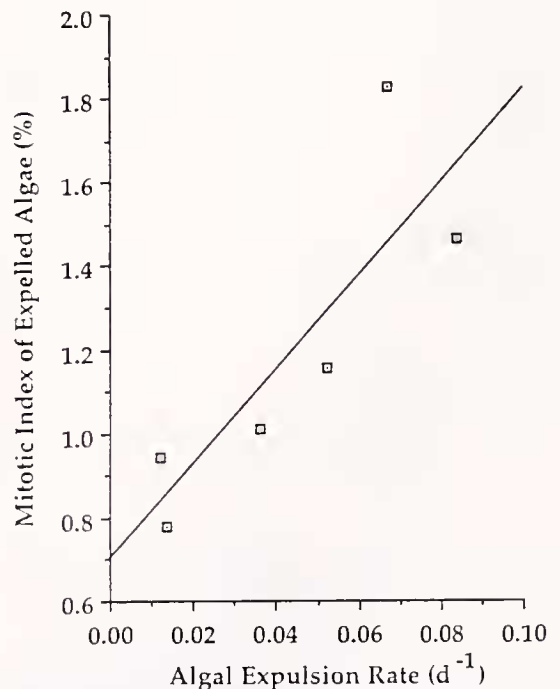


Figure 4. Correlation between mitotic index and rate of expulsion of *Symbiodinium pulchrorum*. $Y = 0.69971 + 11.185X$; $R^2 = 0.711$.

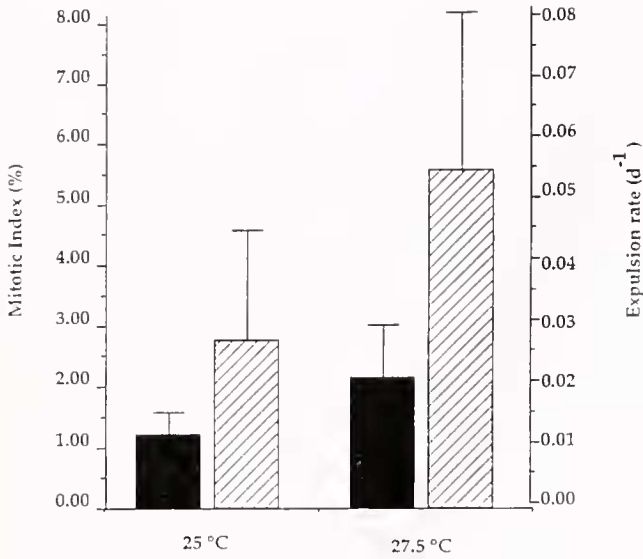


Figure 5. Effect of a 2.5°C temperature increase on rate of expulsion (▨) and mitotic index (■) of *Symbiodinium pulchrorum* released from the symbiotic system. Error bars represent standard deviations of the mean.

expulsion rate and MI, observed in *A. pulchella*, appears to hold only for *P. damicornis* (Fig. 8a); it does not hold for *P. compressa*, *M. verrucosa*, or *F. scutaria* (Fig. 8b, c, d).

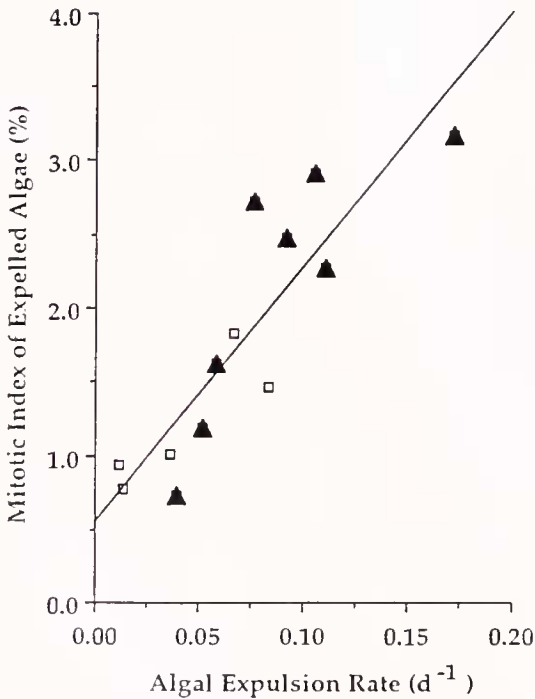


Figure 6. Correlation between mitotic index and rate of expulsion of *Symbiodinium pulchrorum* under control and elevated temperatures. 25°C control (□), 27.5°C experimental (▲). $Y = -0.53328 + 17.244X$; $R^2 = 0.771$.

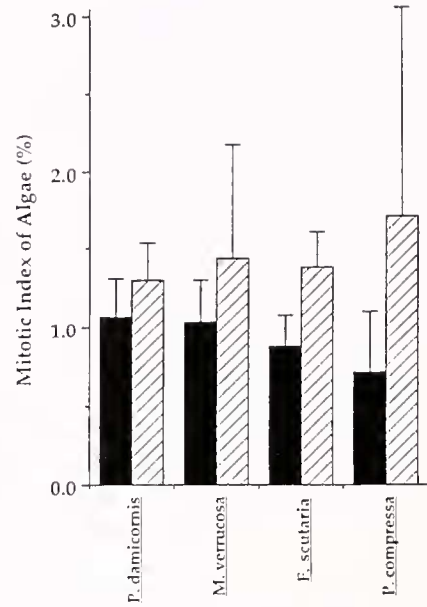


Figure 7. Mitotic indices of expelled algae (▨) and algae remaining within host tissues (■) of different corals, following a 15.5-h incubation at 27°C. Error bars represent standard deviations of the mean.

Discussion

In algal-cnidarian symbioses, regulation of algal numbers is an essential part of the symbiotic relationship, both during “steady state” (Muscatine *et al.*, 1975a, b; Trench, 1987) and during recovery of cnidarians from “bleaching events” (Gates, 1990; Hayes and Bush, 1990; Fitt *et al.*, 1993; Jones and Yellowlees, 1997). The present study has established that preferential expulsion of dividing algae contributes to regulation of algal-cnidarian symbiosis. If dividing algal cells are more likely to be expelled from the host, net algal population growth (within their hosts) will be effectively regulated.

Mitotic index of algae expelled naturally versus isolated mechanically

Algae naturally expelled from *Aiptasia pulchella* and other Hawaiian marine cnidarians have a higher MI than the algae remaining in their hosts (Figs. 1, 7). Suharsono and Brown (1992) and McCloskey *et al.* (1996) have also observed this phenomenon in other cnidarian species. Citing the most parsimonious explanation, these studies suggested that the increase in algal division rates is perhaps due to a lack of host regulation in the released algae. The present study tests this hypothesis by addressing an alternative hypothesis—that the higher MI of the released algae could be explained by preferential expulsion of dividing algal cells. These two possibilities were tested by separating the algae from the host and observing any changes in the algal MI. If the increase in MI is simply a function of a lack of host regulation, then artificially releasing the algae should result in an increase in MI. However, algae artificially

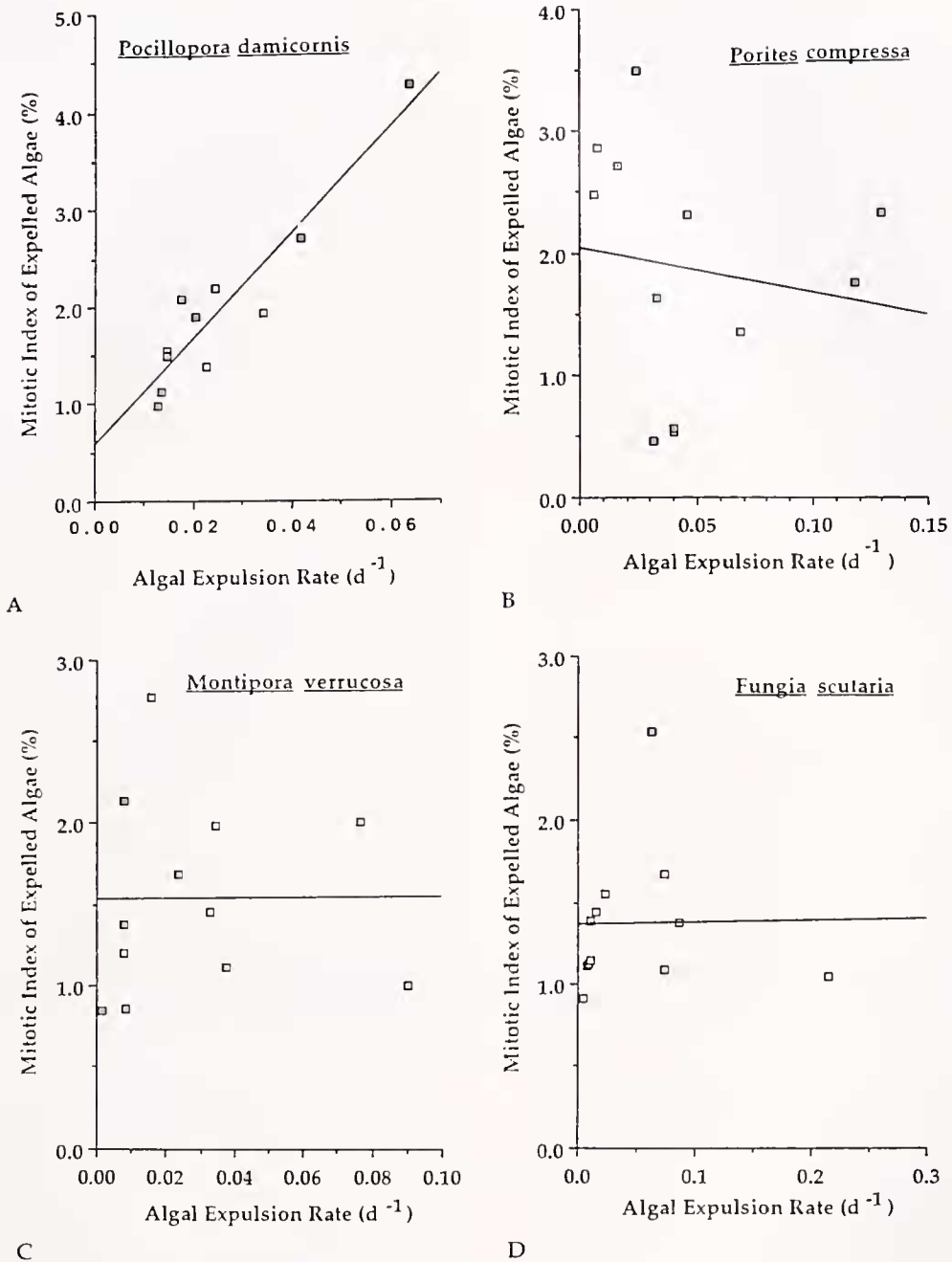


Figure 8. Correlation between mitotic index and rates of expulsion for various hermatypic corals from Kaneohe Bay, Oahu, Hawaii. (A) *Pocillopora damicornis* ($Y = 0.57457 + 54.019X$; $R^2 = 0.869$). (B) *Porites compressa* ($Y = 2.0435 - 3.7634X$; $R^2 = 0.023$). (C) *Montipora verrucosa* ($Y = 1.5287 + 0.12587X$; $R^2 = 0.000$). (D) *Fungia scutaria* ($Y = 1.3582 + 0.11192X$; $R^2 = 0.000$).

released from their host sea anemones (*A. pulchella*) in this study showed no increase in MI.

Incorporation of ³H-thymidine by algae in situ

Uptake and incorporation of ³H-thymidine is an indicator of cells advancing through S-phase of the cell cycle. In *A. pulchella*, cells naturally lost from the symbiosis (following

a preincubation in ³H-thymidine) incorporate more ³H-thymidine (just prior to release) than did cells remaining in the hosts (Fig. 2). These data suggest that the released algae had been in S-phase during the ³H-thymidine incubation and, by inference, were growing and dividing. Therefore, it can be concluded that dividing cells are preferentially expelled from the population.

This is the first study to successfully employ ^3H -thymidine as an indicator of algal division in symbiotic dinoflagellates. Cheney (1974) observed uptake of ^3H -thymidine by the host cells of the coral *Pocillopora damicornis*, but not by the symbiotic algae. Absence of ^3H -thymidine in the algae may have been due to (1) very low growth rates of the resident algae, resulting in minimal uptake of the ^3H -thymidine; (2) uptake and retention of the ^3H -thymidine by the host, thus minimizing availability of ^3H -thymidine to the algae; or (3) a high percentage of substitution of the base thymine by 5-hydroxymethyluracil in the algal DNA (Blank *et al.*, 1988; Taylor, 1990), resulting in low affinity for the ^3H -thymidine molecules in *P. damicornis*. The success of the present experiment may be attributed, in part, to the application of longer incubation times or higher doses of ^3H -thymidine. ^3H -thymidine is a tool that, in parallel with more classic approaches, may be useful in studying regulation of algal-cnidarian symbiosis; in this case, it has provided evidence for preferential loss of dividing algae from symbiotic cnidarians.

Mitotic index, algal expulsion, and effect of temperature

Figure 4 shows a direct linear correlation between expulsion and division rates of algae. That is, higher rates of division translate into greater expulsion of algae, supporting our suggestion of preferential loss of dividing cells (Fig. 3). Further, small increases in temperature, which result in slightly higher rates of division, also result in greater expulsion of algae (Fig. 5). This increased expulsion follows the same linear correlation patterns (relative to division rate) observed previously (Fig. 6). The data therefore strongly support the interpretation that expulsion of algae is preferential and related to the position of the algal cells within the cell cycle.

Expulsion of algae as a mechanism for regulating algal-cnidarian symbiosis

The mechanism involved in preferential expulsion of dividing cells is not clear, but appears to be related to the host's ability to accommodate algal growth. Whereas the expulsion rate of algae from *A. pulchella* in "steady-state" symbiosis is about 0.046 d^{-1} , expulsion of algae from re-infected aposymbiotic anemones during log phase repopulation is negligible (pers. obs.), confirming a previous study on repopulation of bleached individuals of the coral *Acropora formosa* (Jones and Yellowlees, 1997). Therefore, it appears that algal cells are primarily expelled from the system when the host cells can no longer accommodate them. This process can act as a "fine tuning" mechanism for regulating a steady-state symbiosis, where expulsion of algae may be viewed as an inverse function of the host's ability to accommodate new algal cells. Further, this mechanism could explain the differential expulsion of algae from

tentacle *versus* body regions of the cnidarians, where differences in MI and algal densities would require different rates of expulsion from those regions (Muller-Parker and Pardy, 1987).

Preferential expulsion of dividing cells can also play a stabilizing role in algal-cnidarian symbiosis by dampening the effects of environmental conditions that can influence algal division rates. For example, whereas large increases in temperature can lower algal photosynthetic capacities, small increases in temperature may increase algal photosynthesis, metabolism, and thus growth and rates of division (Iglesias-Prieto *et al.*, 1992). However, if rates of algal expulsion vary as a function of the environmentally induced changes in rates of division (Fig. 6), then effectively, by releasing the "excess" cells, the total number of algae within the host will be regulated.

Preferential expulsion of dividing cells is not, however, the only means of regulating algal-cnidarian symbiosis. In case of the green hydra symbiosis, studies have already shown that algal ejection is not the normal mechanism for regulating population densities (McAuley, 1982), probably because of the higher numbers and growth rates of algae per host cell. Among Hawaiian anthozoans, although *A. pulchella* and *P. damicornis* do use preferential expulsion of dividing cells as a regulatory mechanism, other cnidarian species, such as *P. compressa*, *M. verrucosa*, and *F. scutaria*, may not (Fig. 8). This interspecific variability could be a function of the magnitude of "normal" algal growth rates at steady-state for each of the different species considered. Species with higher steady-state growth rates might be more likely to depend on a system of preferential expulsion of dividing cells for regulating their symbiosis. In contrast, among species that have very low daily rates of algal expulsion (such as *Xenia macrospiculata*, *Heteroxenia fuscescens*, *Stylophora pistillata*, and *Millepora dichotoma*; Hoegh-Guldberg *et al.*, 1987), the incidence of expulsion of algae would not significantly affect the regulation of population dynamics. Another possibility is that cnidarians with different algal clades may have evolved different mechanisms of regulation based on sensitivity to environmental factors or on the physiology of their hosts (Rowan *et al.*, 1997). In the case of *M. verrucosa*, *P. compressa*, and *F. scutaria*, the higher MI values of the expelled algae relative to those remaining in the hosts (Fig. 7) may simply be due to a lack of host regulation of division following algal release (Suharsono and Brown, 1992; McCloskey *et al.*, 1996).

A complete understanding of the role of expulsion of algal cells in regulating algal-cnidarian symbiosis requires further research into the release mechanisms involved. This study has shown that at least some symbiotic cnidarians preferentially expel dividing algal cells. In combination with other regulatory factors, this pattern of cell loss can

play a major role in regulating algal-cnidarian symbiosis in steady state.

Acknowledgments

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